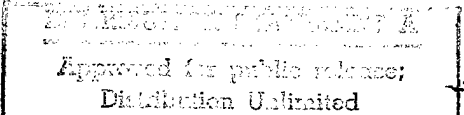


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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 1, 1998	3. REPORT TYPE AND DATES COVERED Progress Report; 6/01/97-6/01/98		
4. TITLE AND SUBTITLE Investigation of Immune Function in Naval Marine Mammals		5. FUNDING NUMBERS Grant N00014-96-1-0619		
6. AUTHOR(S) Tracy A. Romano, PhD				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Texas A&M University Dept. Veterinary Anatomy & Public Health Room 107, VMA Building College Station, Texas 77843		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research Regional Office San Diego 4520 Executive Drive Suite 300 San Diego, California 92121-3019		10. SPONSORING/MONITORING AGENCY REPORT NUMBER		
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12a. DISTRIBUTION/AVAILABILITY STATEMENT  Approved for Public Release				
13. ABSTRACT (Maximum 200 words) Tremendous progress has been made over the past year in the development of cetacean-specific reagents to investigate immune function in Navy marine mammals. We have focused most of our efforts in studying and developing molecular reagents and antibodies to CD4, a cell surface recognition molecule present on T helper lymphocyte that is critical for the immune response. Previously, we cloned cetacean CD4 from a beluga cDNA library using the polymerase chain reaction and cDNA library screening techniques. To generate antibodies against cetacean CD4, we synthesized peptides to different regions of the molecule and expressed the CD4 protein in a bacteria expression vector, and subsequently, injected coupled peptides or the purified CD4 protein into rabbits. Both sets of antibodies were affinity purified, and characterized by Western blot analysis and flow cytometry. These antibodies recognized beluga and bottlenose dolphin lymphocyte lysates by Western blot analysis, but did not recognize the native form of the CD4 protein on the cell surface by flow cytometry. Immunofluorescence on tissue sections is currently being tested with these antibodies. In addition we have generated and purified antibodies to a variant form of CD4 we found in beluga when cloning the full-length CD4. The significance of this potential splice variant of CD4 in the beluga is currently being investigated with the antibodies we have generated.				
14. SUBJECT TERMS				15. NUMBER OF PAGES 4
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17. SECURITY CLASSIFICATION OF REPORT unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT unclassified	20. LIMITATION OF ABSTRACT UL	



## TEXAS A&M RESEARCH FOUNDATION

409-845-8600 409-845-7143 FAX  
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July 17, 1998

Dr. Robert Gisiner, ONR 341BST  
Program Officer  
Office of Naval Research  
Ballston Centre Tower One  
800 North Quincy Street  
Arlington, VA 22217-5660

Reference: Grant No. N00014-96-1-0619  
(432741-00001)

Dear Dr. Gisiner:

In accordance with the Report and Reports Distribution requirements, enclosed are three (3) copies and one (1) diskette of the annual technical report. June 01, 1997 through June 01, 1998.

If additional technical information is required, please contact me at (409)845-5640 or e-mail [jdb@rf-mail-ramu.edu](mailto:jdb@rf-mail-ramu.edu).

Sincerely,

Joan Birdwell  
Research Administrator  
Contracts & Grants

Enclosures

- c: L. Washington, ONR Regional Office (1 copy of SF298only)  
Director, Naval Research Laboratory (1 copy)  
Defense Technical Information (2) ✓  
T. Romano, Principal Investigator (w/o enclosure)

ANNUAL PROGRESS REPORT

Grant#: N00014-96-1-0619

PRINCIPAL INVESTIGATOR: Tracy Romano

INSTITUTION: Texas A&M University

EMAIL: tromano@scripps.edu

GRANT TITLE: Investigation of Immune Function in Naval Marine Mammals

REPORTING PERIOD: 1 June 1997 - 31 May 1998

AWARD PERIOD: 1 June 1996 - 31 May 1999

OBJECTIVE: To generate cetacean-specific immunological reagents in order to investigate immune function in Navy marine mammals

APPROACH: We have generated molecular reagents and antibodies specifically to cetacean lymphocyte markers by utilizing techniques in molecular biology and monoclonal and polyclonal antibody production. Primarily we have focused on the cell surface molecule CD4, which is critical for generation of the immune response. CD4 was cloned from a white whale, Delphinapterus leucas, thymus cDNA library using the polymerase chain reaction and cDNA library screening. Antibodies were raised to different peptide fragments of the whale CD4 molecule, as well as to the expressed CD4 protein itself. These antibodies have been characterized by ELISA, Western blot analysis, and flow cytometry.

ACCOMPLISHMENTS (last 12 months): Most of our efforts over the past 12 months have focused on the production of antibodies to whale CD4. Antibodies to whale CD4 and the variant form of CD4 we found in whale (reported last year), were produced by synthesis of peptide fragments to the whale CD4 molecule, and also expression of whale CD4 protein in a bacterial expression system. The resultant peptides were coupled to keyhole limpet hemocyanin, and injected into rabbits, while the expressed proteins were purified and injected into rabbits for antibody production. Each antibody was purified over a protein G column and subsequently passed over an affinity column prepared for each individual peptide. The antibodies were tested by immunofluorescence on whale tissue sections, flow cytometry on whale peripheral blood lymphocytes, and Western blot on whale lymphocyte lysates. The antibodies work by Western blot, recognizing an approximate 65 kilodalton protein in beluga lymphocyte lysates and an approximate 60 kilodalton protein in bottlenose dolphin lymphocyte lysates. However, none of the antibodies recognized the native CD4 protein on the cell surface by flow cytometry. Expressed protein antibodies are currently being tested by immunofluorescence.

SIGNIFICANCE: The study of CD4 in cetaceans is valuable for clinical monitoring and health assesement of cetaceans kept under the Navy's care as well as for those in the wild. The CD4 reagents we presently have as well as an antibody that will recognize the native protein, (which we are currently working on), will enable us to quantify baseline levels of CD4 in Navy dolphins and whales and provide information as to

how these levels change during sickness, transport, and routine and novel exercises. We will also be able to label CD4-positive lymphocytes in the lymphoid organs of hunted, stranded, and/or expired animals, and determine lymphocyte targets of postganglionic nerve fibers of the autonomic nervous system in primary and secondary lymphoid organs.

The study and comparison of whale CD4 is significant in itself given the importance of this molecule in the immune response, its interaction with major histocompatibility class II molecules, and since it is the receptor for the human immunodeficiency virus. Comparison of whale CD4 with other species has revealed interesting comparative information in regards to the evolution and adaptation of the mammalian immune system (reported in the recently submitted manuscript entitled: "Molecular cloning and characterization of CD4 in an aquatic mammal, the white whale, *Delphinapterus leucas*", by Romano et al.).

WORK PLAN (Next 12 months): The major objective of the next 12 months work is to produce a monoclonal antibody that recognizes the native CD4 protein. The antibodies we have for use in Western blots will be important for health assesment of Navy cetaceans; however, it will be important to actually be able to visually see which cells in the blood and tissues are CD4 positive. In order to try to obtain an antibody against the native CD4 protein, we will clone cetacean CD4 into a mammalian expression vector, transfect into a mouse cell line, and inject CD4 expressing cells into mice for monoclonal antibody production.

In addition, we will finish characterization of the CD4 antibodies we have by working out optimal conditions for immunofluorescence on tissue sections. Further investigation will also continue on the variant form of CD4. The antibodies we have will be tested on lymphocytes maintained in culture and their supernatant, as well as whale and dolphin serum. Northern blot analysis will be carried out on various regions of the whale brain as well, since a variant form of CD4 has been found in the mouse and human brain.

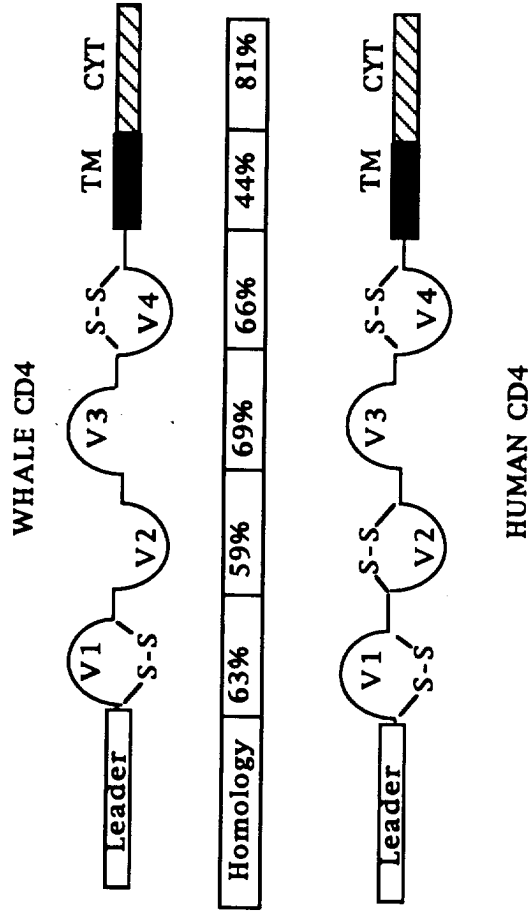
PUBLICATIONS AND ABSTRACTS (last 12 months):

Romano, T.A., S.H. Ridgway, D.L. Felten, and V. Quaranta. (1998) Molecular cloning and characterization of CD4 in an aquatic mammal, the white whale, *Delphinapterus leucas*. Submitted to *Immunogenetics*

Romano, T.A., J.A. Olschowka, S.Y. Felten, V. Quaranta, S.H. Ridgway, and D.L. Felten. (1998) Immune response, stress, and environment: Implications for cetaceans. In: Cell and Molecular Biology of Marine Mammals. C.J. Pfeiffer (ed) (should be published in '98)

Romano, T.A., S.H. Ridgway, D.L. Felten, and V. Quaranta. (1998) Investigations of the cetacean immune system: Molecular cloning of beluga whale CD4. In: Society for Marine Mammalogy Abstracts/World Marine Mammal Conference, Monaco pp.116. (Abstract and presentation)

Romano, T.A., D.L. Felten, S.H. Ridgway, and V. Quaranta. (1998) Investigations of the Cetacean Immune System. In: XXII Reunion Internacional para el estudio de los Mamiferos Marinos Abstracts, Quintana Roo, Mexico, pp.52. (Invited abstract and presentation)



## Objectives

- Clone whale lymphocyte cell surface marker, CD4
- Express whale CD4 and develop whale-specific CD4 antibodies
- Use molecular CD4 reagents, and whale-specific antibodies to monitor and assess health in Navy dolphins and whales

## Accomplishments

- Cloning of whale CD4
- Expression of whale CD4 and generation of whale-specific antibodies
- Comparison of whale CD4 sequence and structure with other mammals

## Significance

- CD4 is important for the generation of an immune response and immunocompetence
- CD4 whale-specific reagents will aid in clinical monitoring and health assessment of Navy dolphins and whales
- Comparison of whale CD4 with other mammals reveals information on the evolution and adaptation of the immune system

ANNUAL REPORT QUESTIONNAIRE  
(for ONR use only)

Principal Investigator: Tracy Romano, PhD  
Institution: Texas A&M University  
Project Title: Investigation of Immune Function in Naval Marine Mammals

Number of ONR supported

Papers published in refereed journals: 1  
Papers or reports in non-refereed publications: 2  
Books or book chapters published: 1

Note: The manuscript and book chapter will be published in 1998

Number of ONR supported patents/inventions

Filed: \_\_\_\_\_  
Granted: \_\_\_\_\_  
Patent name(s) and number(s): \_\_\_\_\_

HAVE YOU LICENSED TECHNOLOGIES (E.G., SOFTWARE) THAT WERE DEVELOPED  
WITH ONR SUPPORT? IF SO, PLEASE DESCRIBE ON A SEPARATE SHEET.

No

HAVE YOU DEVELOPED INDUSTRIAL/CORPORATE CONNECTIONS BASED ON YOUR ONR  
SUPPORTED RESEARCH? IF SO, PLEASE DESCRIBE ON A SEPARATE SHEET.

No

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Trainee Data (only for those receiving full or partial ONR support):

TOTAL	FEMALE	MINORITY	NON-US CITIZEN
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No. Grad. Students:

No. Postdoctorals:

No. Undergraduates:

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AWARDS/HONORS TO PI AND/OR TO MEMBERS OF PI'S RESEARCH GROUP (please describe):

1. Invited speaker to the XXII Reunion Internacional Mamiferos Marinos
2. Session Chair for the IAAAM meeting held in San Diego 5/98

Equipment purchased on grant (number and description of items costing  
>\$1,500):

1. Nikon TMS-F Inverted Microscope \$5847.16
2. Pharmacia Biotech Ultrospec 1000 \$3995.00